NUTRIENT UPTAKE, GROWTH, PROTEIN PRODUCTION AND COBALAMIN ACCUMULATION OF *EUGLENA GRACILIS* IN BAKER'S YEAST PRODUCTION WASTEWATER

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PURPOSE AND GOALS

Microalgae are unicellular eukaryotic organisms capable of photosynthesis. They harvest sunlight and efficiently take up carbon dioxide and nutrients such as nitrogen and phosphorus from their environment and use them for their growth. Due to these properties, their rapid growth and ability to survive in a variety of environments, microalgae have potential in biotechnological applications that promote nutrient recovery and recycling, water purification and the carbon neutral production of biochemicals and possibly biofuels.

The purpose of this study was to investigate the suitability of a side stream water originating from the production of baker's yeast (yeastwater) for the cultivation of a species of microalga called *Euglena gracilis*. The study aimed to determine the capacity of this water to support growth and protein production of *E. gracilis* as well as the capacity of *E. gracilis* to remove nutrients from the water. The effect of filtration of the water on these parameters was also studied. Yeastwater contains an organic molecule called betaine in relatively high concentrations. Betaine has previously been shown to boost the production of the important vitamin cobalamin in bacteria. The study aimed to determine the effect of betaine on the growth of *E. gracilis* and on the production of cobalamin in the algal-bacterial symbiosis.

METHODS

E. gracilis was cultured in laboratory scale photobioreactors. Its growth, protein production and nutrient uptake capacity was determined. Baker's yeast production side stream water diluted with MQ-water was used as the growth medium either in filtered or unfiltered form. A control treatment was prepared where no microalgal inoculate was added to the photobioreactor. The same microalga was also grown in a synthetic nutrient medium with and without betaine. The uptake of betaine and biomass concentrations of cobalamin were determined. For the determination of microalgal growth, dry weight determination and flow cytometry analysis were used. Protein production was determined on the basis of total nitrogen concentration in the biomass. Spectrophotometric measuring kits were used for the determination of nutrient concentrations. Liquid chromatography techniques were used for the determination of betaine and cobalamin concentrations.

RESULTS

Significant microalgal growth was observed in filtered yeastwater, while growth in unfiltered yeastwater was very low. Nitrogen removal was higher in presence of *E. gracilis* compared to the control treatment. Protein production in yeastwater was comparable to that of microalgae grown in synthetic medium. *E. gracilis* grew much better in the synthetic media supplemented with betaine than without the addition. Betaine enrichment had no effect on cobalamin production. Cobalamin was produced in unfiltered yeastwater both with and without the presence of *E. gracilis*.

CONCLUSIONS

Unfiltered yeastwater does not support growth of *E. gracilis* possibly due to its high turbidity. Filtered yeastwater, on the other hand can support the production of *E. gracilis* biomass. *E. gracilis* can be used to reduce nitrogen concentrations in yeastwater. Yeastwater can support cobalamin production by bacteria, but this phenomenon did not benefit from the presence of the microalga. The effect of betaine on microalgal growth warrants further study to determine whether it is related to the accumulation of intracellular nutrients, storage compounds or to some other phenomenon. Yeastwater is a promising nutrient feedstock for microalgal biomass production. However, the role of filtration and possibility of using other methods for turbidity reduction needs to be further studied.

Avainsanat – Nyckelord – Keywords
Microalgae, wastewater, sidestream, betaine, cobalamin, protein

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TARKOITUS JA TAVOITTEET

Mikrolevät ovat yksisoluisia tumallisia eliöitä, jotka kykenevät fotosynteesiin. Ne käyttävät auringon valoenergiaa, hiilidioksidia ja ravinteita, kuten typpeä ja fosforia, kasvuunsa. Näiden ominaisuuksien lisäksi mikrolevien nopea kasvu ja kyky selviytyä monissa erilaisissa ympäristöissä tekee niistä ihanteellisia ehdokkaita bioteknologian työkaluiksi. Niiden avulla voitaisiin edistää ravinteiden talteenottoa ja kierrätystä, vedenpuhdistusta ja biomolekyylien sekä mahdollisesti biopolttoaineiden hiilineutraalia tuotantoa.

Tässä tutkielmassa selvitettiin leivinhiivan tuotantoprosessista peräisin olevan sivuvirtaveden (emäveden) soveltuvuutta *E. gracilis* -mikrolevän kasvualustaksi. Tarkoitus oli tutkia mikrolevän kasvua ja proteiinintuotantoa emävedessä, sekä mikrolevän kykyä poistaa ravinteita emävedestä. Tutkimuksessa haluttiin myös selvittää emäveden suodatuksen vaikutusta *E. gracilis* -mikrolevän kasvuun. Emävedessä on betaiiniksi kutsuttua orgaanista yhdistettä varsin runsaasti. Aikaisemmissa tutkimuksissa sen on havaittu parantavan tärkeän kobalamiinin (vitamiini B12) tuotantoa bakteereissa. Työssä tutkittiin myös betaiinin vaikutusta mikrolevän kasvuun ja mikrolevä-bakteeri -yhteisön kykyyn tuottaa kobalamiinia.

MENETELMÄT

Tutkimuksessa kasvatettiin *Euglena gracilis* -mikrolevää laboratorioskaalan fotobioreaktoreissa. Sen kasvu, proteiinin tuotanto ja kyky poistaa ravinteita selvitettiin. Kasvualustana käytettiin MQ-vedellä laimennettua emävettä joko suodatettuna tai suodattamattomana. Lisäksi suoritettiin kontrollikäsittely, jossa fotobioreaktoreita ei siirrostettu mikrolevällä. Toisessa kokeessa samaa mikrolevää kasvatettiin keinotekoisessa ravinneliuoksessa ilman betaiinia ja sen kanssa. Betaiinin poistuma ja kobalamiinin tuotanto määritettiin. Levän kasvua tutkittiin kuivapainomääritysten ja virtaussytometristen analyysien keinoin. Proteiinintuotanto määritettiin levämassan typpipitoisuuden perusteella. Ravinnepitoisuudet määritettiin spektrofotometrisesti. Betaiinin ja kobalamiinin pitoisuudet määritettiin nestekromatografian keinoin.

TULOKSET

E. gracilis kasvoi varsin hyvin suodatetussa emävedessä, mutta selvästi heikommin suodattamattomassa emävedessä. Kontrollikäsittelyyn verrattuna mikrolevä edisti typenpoistoa emävedestä. *E. gracilis* -mikrolevän proteiinintuotanto emävedessä oli verrattavissa keinotekoisella kasvualustalla kasvaneen levän proteiinintuotantoon. Betaiinin lisäys keinotekoiseen kasvualustaan paransi mikrolevän kasvua huomattavasti, mutta kobalamiinin tuotantoon se ei vaikuttanut. Suodattamattomassa emävedessä syntyi kobalamiinia riippumatta siitä, oliko siinä mikrolevää vai ei.

JOHTOPÄÄTÖKSET

Suodattamaton emävesi ei sovellu mikrolevän kasvatukseen mahdollisesti sen suuren sameuden vuoksi. Suodatettu emävesi sen sijaan soveltui *E. gracilis* -levän kasvatukseen, ja mikrolevää voidaan käyttää emäveden typpipitoisuuden alentamiseen. Emävedessä on mikrobitoimintaa, joka tuottaa kobalamiinia, mutta tuotanto ei ole riippuvaista mikrolevän läsnäolosta. Betaiinin vaikutusta mikrolevän kasvuun on syytä tutkia lisää, jotta voidaan selvittää perustuuko sen vaikutus ravinteiden tai varastoaineiden kerääntymiseen mikroleväsoluun vaiko johonkin muuhun ilmiöön. Emävesi on lupaava ravinnelähde mikrolevien kasvatukseen, erityisesti silloin, kun tarvitaan typpirikasta raaka-ainetta muiden ravinnelähteiden lisäksi ja erityisesti, jos löydetään tapoja tehokkaasti alentaa emäveden sameutta.

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1. Introduction

1.1 The role of microalgae in circular economy

Currently prevailing systems of industrial production in most societies are unsustainable. They contribute to climate change, the depletion of natural resources and the degradation of natural ecosystems, which in turn, human societies are dependent on for a multitude of ecosystem services. It is widely acknowledged that, to ensure the health of Earth's biosphere and future generations' access to natural resources, the global economy must be made more resource efficient. This objective is included in the United Nations sustainable development goals (Rosa 2017).

One of the paths to better resource efficiency is a better exploitation of resources which are presently considered waste or otherwise not exploited to their full potential. One such class of material is organic waste. Different types of organic waste are produced in many industrial sectors and often contain significant amounts of residual chemical energy and nutrients. In particular the food industry generates a considerable amount of organic waste, which has great potential as feedstock for a variety of bio-based products (Dahiya *et al.* 2018). At present it is mostly used to produce low value products such as biogas, animal feed, compost or other agricultural fertilizers. Further research is needed to discover ways to better valorise this waste fraction.

Microalgae are unicellular, eukaryotic organisms capable of photosynthesis. They are important primary producers in Earth's biosphere and represent a very high biodiversity. Microalgae have great potential in wastewater treatment since they efficiently take up nutrients from their environment. They use the energy of solar radiation and capture carbon dioxide (CO_2), making them ideal candidates for applications in carbon neutral technologies. In addition to photoautotrophic growth where cells harvest energy from sunlight, many microalgae are also capable of heterotrophic growth where they use organic carbon present in their growth medium, or a combination of the two (mixotrophy) (Lowrey *et al.* 2015).

Thus, microalgae have potential to use the nutrients and carbon found in organic waste, preventing their release into the environment and helping to recycle them into new products. For algae that are capable of mixotrophy, the presence of an organic carbon source may enhance the growth rate of the culture and reduce the need for an external CO_2 supply (Li *et al.* 2014). This will, in turn, reduce biological and chemical oxygen demand (BOD, COD) and therefore the polluting potential of the water used as culture medium. On the other hand, the effects of an organic carbon source on microalgal growth depend on many factors and can be also inhibitory (Cerón García *et al.* 2005).

Integration of microalgae production with existing industrial plants would have several benefits such as the use of waste heat and CO_2 as well as the recycling of water and nutrients, all of which would reduce the ecological footprint of the industrial plant. Traditionally, microalgae are cultivated as pure cultures as far as is technically possible. However, most waste waters contain existing micro-organisms and their sterilization could be problematic from a cost perspective. Fortunately, microalgae can be cultivated in the presence of bacteria as well. This may, depending on the interactions between the organisms, either inhibit or promote the growth of the algae (Guo & Tong 2014; Han *et al.* 2016). In an algal-bacterial co-culture, the bacteria degrade organic compounds, producing CO_2 that the algae can use in photosynthesis. The algae in turn produce oxygen which the bacteria need for their degradation activity (Subashchandrabose *et al.* 2011). This kind of culture removes not only nutrients but also reduces the BOD of the wastewater (Safonova *et al.* 2004). The need for external supplies of CO_2 or oxygen are also reduced in such a system. The oxygen supplied by the algae can also be used by ammonium oxidising bacteria, further contributing to nitrogen removal from the wastewater (Wang *et al.* 2015b).

Another argument in favour of using microalgae for the purification of waste effluents is the fact that many of the biomolecules they produce are of higher value than the end-products of conventional waste water treatment technologies such as anaerobic digestion or composting. Utilization of waste streams as growth substrates for microalgae can therefore make the

exploitation of different types of waste more economically viable and reduce the need for raw materials made from virgin sources.

Some of the biomolecules that microalgae produce are valuable compounds such as proteins, lipids, pigments and antioxidants. Several species are producers of omega-3 fatty acids such as EPA and DHA necessary for human nutrition (Adarme-Vega *et al.* 2012). Microalgal biomass generally has a high protein content and its protein is of high quality (containing all essential amino-acids) considering human nutritional requirements (Becker 2007). It can therefore be one of the solutions for the growing demand for sustainably produced protein. Especially considering that the cultivation of microalgae does not compete with resources needed for conventional agricultural crops and that the cultivation of microalgae requires less surface area per unit of biomass produced than conventional crops (Draaisma *et al.* 2013).

1.2 Potential of *Euglena gracilis* in biotechnology

Euglena gracilis G. A. Klebs is a flagellate freshwater microalgae strain, which can grow in wastewater (Mahapatra *et al.* 2013). It is capable of autotrophy and mixotrophy and therefore can take advantage of some organic compounds present in its cultivation medium. This can enhance its biomass productivity (Fujita *et al.* 2008). This metabolism of organic carbon by microalgae also contributes to the removal of organic carbon and of oxygen depletion potential from the wastewater (Wang *et al.* 2015a).

E. gracilis tolerates low pH (Yamane *et al.* 2001) which can be an advantageous trait for avoiding contamination of its cultures by other micro-organisms. It has a variety of metabolic capabilities (O'Neill *et al.* 2015) and potential biotechnological applications (Krajčovič *et al.* 2015). It can produce large quantities of the antioxidant α-tocopherol (vitamin E) (Ogbonna *et al.* 1998), several B-vitamins (Baker *et al.* 1981) and ascorbic acid (vitamin C) (Shigeoka *et al.* 1979), as well as β-carotene (Threlfall & Goodwin 1967) which acts as a precursor of

vitamin A. It also produces a β-glucan called paramylon (Grimm *et al.* 2015). Cells of *E. gracilis* lack a cellulosic cell wall, making it easier to digest or further process its biomass.

1.3 Cobalamin

Cobalamin, also known as vitamin B12 is an essential micronutrient for humans. It is not produced by any eukaryotes (Warren *et al.* 2002). Many animals have cobalamin producing micro-organisms in their digestive tract, while vascular plants do not require it for their metabolism. Humans fulfil their requirement of cobalamin by consuming animal products, making deficiency a risk for those following a vegan diet (Pawlak *et al.* 2014). Synthetization of cobalamin is a complex process involving many steps and its commercial production happens via bacterial fermentation (Martens *et al.* 2002). It is a high value product used in the manufacture of nutritional supplements and fortified foods.

Some cyanobacteria are capable of producing cobalamin, however, most of it consists of a pseudovitamin form of cobalamin, not active in human metabolism (Watanabe *et al.* 1999). Eukaryotic microalgae cannot produce cobalamin, but many species have been found to require it for their metabolism (Croft *et al.* 2005). They obtain cobalamin from mutualistic interactions with symbiotic bacteria (Croft *et al.* 2005). *E. gracilis* requires cobalamin for its growth and accumulates it in its cells (Varma 1961).

1.4 Potential of baker's yeast production wastewater in the cultivation of microalgae

1.4.1 Baker’s yeast production

Baker's yeast (*Saccharomyces cerevisiae)* is a basic raw material of the food industry. The main feedstock for its culture is molasses, a sugar industry product derived either from sugarcane (*Saccharum* sp.) or sugar beet (*Beta vulgaris* var. *altissima*). The production of

baker's yeast generates considerable amounts of wastewater. COD in this water is typically high and it contains high amounts of nitrogen and solids and has a dark colour (Ersahin *et al.* 2011). Therefore, it can cause eutrophication, oxygen depletion and increased turbidity if released into natural waterbodies without treatment. Baker's yeast production wastewater (yeastwater) is presently used as feedstock for anaerobic biogas production or concentrated into vinasse which is used as agricultural fertilizer. Both of these are low value products that do not take full advantage of the characteristics of yeastwater.

To my knowledge, yeast production wastewater has not previously been utilized for microalgal culturing. However, sugar beet vinasse from sugar production has been successfully used for the culturing of cyanobacteria (Coca *et al.* 2015). The microalgae *Chlorella vulgaris* and *Scenedesmus* sp. have been cultured in vinasse originating from the production of ethanol from sugarcane (Candido & Lombardi 2017; Ramirez *et al.* 2014).

Commercialization of wastewater cultured microalgal biomass requires careful consideration and management of potential sources of contamination. Potential contaminations in wastewaters are varying from microbial contamination to heavy metals. The cleanliness requirements will depend on the use of the biomass. High standards are required when it is used as such as an ingredient in foods, while for the production of polymers or biofuels for example more contamination can be acceptable. For growing microalgae to be used in foods, the use of a nutrient feedstock originating directly from a process with food-grade cleanliness standards may offer significant advantages over alternative feedstocks such as those originating from livestock production or municipal wastewater treatment. Yeastwater is one such nutrient feedstock, being high in nutrients and originating from a tightly controlled biotechnological process that results in a food-safe end-product. However, it should be noted that yeastwater can contain yeast cells remaining from the production process as well as other microbia from the wastewater holding tanks.

1.4.2 Betaine

When sugar beet molasses is used in baker's yeast production, the wastewater also contains significant amounts of trimethylglycine (glycine betaine) originating from the sugar beets used for manufacturing the molasses (Ersahin *et al.* 2011). Trimethylglycine or glycine betaine (called simply betaine in the rest of the text) is a non-proteogenic amino acid. In cellular metabolism it acts as a methyl-group donor and osmoregulator (Craig 2004). It was first isolated from sugar beet, which contains betaine in high concentrations. Betaine has been shown to enhance plant stress tolerance (Ashraf & Foolad 2007). It has also shown promise in boosting cellular metabolism and cobalamin productivity in certain species of bacteria used for commercial scale cobalamin production (Li *et al.* 2013). It has been used to boost microalgal growth under suboptimal temperatures (Wang *et al.* 2016).

1.5 Aims of this study

In this study, *E. gracilis* was grown in yeast production wastewater, filtered yeast production wastewater and a synthetic growth medium containing betaine. Yeastwater filtration was expected to improve the growth of *E. gracilis* by reducing turbidity and therefore improving light penetration, and by removing a portion of competing micro-organisms, mainly yeasts. A control treatment was prepared with yeastwater without algal inoculate. A non-axenic strain of *E. gracilis* was used for the experiments. The yeastwater was not sterilized for the experiments, therefore retaining its microbial population. Reduction of nutrient concentrations in yeast production wastewater was measured to determine the capacity of *E. gracilis* to purify this water. The biomass productivity of the alga was determined, and the produced algal biomass was analysed for its concentrations of protein, cobalamin and betaine. An experiment in synthetic growth media was also carried out to determine the impact of betaine on the metabolism of cobalamin. This included a control experiment in synthetic growth medium without the addition of betaine.

This study tries to answer the following questions: 1) Does *E. gracilis* reduce the nutrient concentrations and COD of baker's yeast production wastewater? 2) Does simple filtration make the wastewater more suitable as a growth medium for *E. gracilis*? 3) What is the effect of betaine on the growth of the algal culture? 4) Does the presence of betaine stimulate cobalamin production? 5) Are betaine and cobalamin accumulated in algal biomass grown in yeastwater? 6) Does the use of baker's yeast production wastewater support protein production comparably to a traditional culture medium?

2. Materials and methods

2.1 Growth experiment

The algal strain used in the growth experiment was *E. gracilis* CCAP 1224/5Z. It was grown in a non-axenic stock culture. Prior to the experiment, the alga was pre-cultured in two cylindrical glass bottles of 4 L and 1.8 L liquid volume each in modified Hutner's medium (Hutner *et al.* 1966) (see Appendix A for composition). In this study, five different growth experiments were performed with four different growth media using four replicates. Growth of *E. gracilis* was studied in diluted yeastwater (10 % of yeastwater), in filtered and diluted (10%) yeastwater, as well as in a control experiment in diluted yeastwater (10 %) without any algal inoculate. The purpose of the control was to differentiate between the effects of the alga and the natural microbial community of the yeastwater on the studied phenomena. The dilution percentage was chosen based on preliminary experiments where higher concentrations of yeastwater had failed to support the growth of *E. gracilis* (not reported here). The effect of added betaine on algal growth was studied in experiments in modified Hutner's medium; one experiment in medium containing cobalamin and lacking betaine, and one in medium lacking cobalamin but containing betaine. Yeast production wastewater (yeastwater), originating from the yeast dewatering process was acquired from Suomen Hiiva Oy (Lahti, Finland) in the morning and the growth experiment was started during the same day.

For preparation of the filtered yeastwater growth medium, yeastwater was first centrifuged (Heraeus Multifuge 1 S-R, Kendro Laboratory Products, Germany) at 4234×*g* for 5 minutes to remove a portion of the solids and then filtered through glass fibre filters (Whatman GF/C, 90 mm ⌀, nominal particle retention 1.2 µm) with vacuum filtration. Growth media containing yeastwater were prepared by measuring 1350 mL of ultrapure water into the culture bottles and adding 150 mL of yeastwater or filtered yeastwater. For Hutner's medium containing cobalamin, cobalamin was added after autoclaving, sterilizing the cobalamin stock-solution by sterile filtration through 0.22 µm pore size syringe filters (13 mm ⌀, PFTE, VWR, USA). For Hutner's medium containing betaine, 22.3 grams of betaine-hydrochloride was added to each bottle prior to autoclaving resulting in a betaine concentration of 10 g L^{-1}. The yeastwater based media were not sterilized before use, while the two synthetic media were autoclaved. Compositions of the different growth media are shown in Table 1. Exact medium composition for modified Hutner's medium is described in Appendix A.

Table 1. Medium composition in the different experiments

Medium composition	Medium	Abbreviation
10 % yeast production wastewater, 90 % ultrapure water	yeastwater	YW
10 % filtered yeast production waste water, 90 % ultrapure water	filtered yeastwater	YW, filtered
10 % yeast production wastewater, 90 % ultrapure water	yeastwater (control experiment)	YW, control
modified Hutner's medium, 19.85 µg L^{-1} of cobalamin	Hutner's medium	HM
modified Hutner's medium, no cobalamin, 10 g L^{-1} of betaine	betaine enriched Hutner's medium	HM + betaine

Cultivation of the inoculate and the experiment itself was done under a 16:8 light:dark illumination regime in a temperature-controlled growth chamber (Friocell CLC-E / FC 404, MMM Group, Germany) at 25°C temperature. Light was provided by white LED strips. Light

intensity in the chamber was approximately 200 µmol $m^{-2}s^{-1}$, measured with a Li-Cor 190R Quantum Sensor and LI-1400 Light Sensor Logger, (Li-Cor, Lincoln, Nebraska, USA). Cultivation was done until the end of the exponential growth period.

Cylindrical glass bottles with a total volume of 2000 mL were used for all experiments. The culture liquid volume was 1550 mL for all experiments except the betaine enriched Hutner's medium, where, due to pH adjustment (see below), the culture volumes were between 1665 and 1800 mL. For aerating and mixing the culture, air was continuously bubbled into the bottles. The air was drawn from the ambient room atmosphere, bubbled through a bottle filled with water for humidification and then passed into the culture bottles via a 2 mL glass pipette fitted into the bottle through a hole in the bottle cap. A 0.2 µm pore size PFTE membrane filter (Acro 37 TF Vent) was used between the air line and the pipette to avoid contamination by airborne microbia. A second 2 mL glass pipette with a piece of silicone tubing on its upper end was fitted through the bottle cap for the withdrawal of liquid samples. A third hole in the bottle cap allowed the outflow of air through a piece of silicone tubing and another 0.2 µm pore size PFTE filter. The rate of airflow into each bottle was approximately 0.5 L min^{-1}. During the photoperiod, the air was enriched with pure CO_2 (2 % of CO_2 in air). The bottles and bottle cap assemblies had been sterilized in an autoclave at 121°C for 60 minutes prior to use. Before inoculation, pH was measured from all growth media. In betaine enriched Hutner's medium it was under 2 due to the hydrochloride content of the added betaine-hydrochloride. To avoid the influence of pH on the results, it was adjusted to same level as in other treatments (pH 6) by adding 1 molar NaOH solution.

To avoid the presence of inoculate growth medium in the experimental cultures, the algal inoculate was concentrated by centrifugation before adding to the growth bottles. For each growth bottle, 160 mL of inoculate was divided into four centrifuge tubes and gently centrifuged at 134×*g* for 5 minutes (Heraeus Multifuge 1 S-R, Kendro Laboratory Products, Germany). The resulting biomass pellets were pooled into one tube and resuspended into approximately 50 mL of supernatant before pouring into the growth bottles. The initial biomass concentration was 0.30 g L^{-1} dry weight (DW) for all experiments except betaine

enriched Hutner's media, where the concentration was between 0.26 and 0.28 g L^{-1} due to the larger culture volume. The inoculate cell density was 2900 cells μL^{-1}, which lead to an initial concentration of 260–280 cells μL^{-1} in betaine enriched Hutner's medium and 300 cells μL^{-1} in other media. Bottles were placed in the growth chamber in a randomized order and rearranged with each daily sampling to provide equal light distribution to all bottles over the course of the experiment.

2.1.1 Daily sampling and growth monitoring

Liquid samples of 10 mL were withdrawn on each day except the fourth and fifth days of the experiment from each bottle with the help of a 50 mL plastic syringe (HSW SOFT-JECT). The daily samples were used for determination of dry weight (DW), pH and cell concentration. Prior to sampling, the bottles were shaken briefly to resuspend as much of the algal cells as possible. Nevertheless, thin algal biofilms were observed to form on the walls of all bottles.

On days 1, 6, 8 and 9 the DW of the samples was determined. For DW determination, 2–5 mL of sample was filtered on pre-dried and weighed glass fibre filters (Whatman GF/C, 47 mm ⌀, nominal particle retention 1.2 µm) using vacuum filtration equipment. The filters were dried overnight at 105°C, cooled to room temperature in an exicator and weighted. Determination of pH was performed on samples from days 2, 6 and 9 of the experiment using a SI Analytics Blueline pH probe.

A cytometry analysis with a Partec CyFlow CUBE 8 flow cytometer was performed on each sampling day of the experiment to determine cell concentrations in each of the replicates. The excitation wavelength was 488 nm and forward scatter, side scatter as well as fluorescence at 536 nm were recorded. The sample volume was 200 µL and the run speed 4 $\mu L\ s^{-1}$. Samples were diluted 10–100 times with ultrapure water and briefly vortexed prior to analysis. Gating regions for algae, yeast and bacteria were established prior to the experiment

using liquid cultures of *E. gracilis* and *Pseudomonas fluorescens* as well as a suspension of *Saccharomyces cerevisiae* cells. Cell concentrations were measured, not only for *E. gracilis*, but also for bacteria and yeasts with the aim of monitoring the development of the microbial population present in the yeastwater during the experiment.

2.1.2 Harvesting

On day nine of the experiment, when growth in all cultures had ceased, the biomass was harvested. Before harvesting, one last sampling was done after having thoroughly shaken the bottles. The contents of each bottle were transferred into 400 mL centrifuge jars and centrifuged at 1400×*g* for 10 minutes (Heraeus Multifuge 1 S-R, Kendro Laboratory Products, Germany).

After centrifugation most of the supernatant was collected and the remaining algal biomass-supernatant slurry was transferred into 50 mL Falcon tubes for another round of centrifugation (1400×*g*, 10 minutes) after which the remaining supernatant was collected, and the algal biomass was pooled into plastic Falcon tubes. The Falcon tubes with the algal biomass pellets were stored at –20°C. Biomass samples were freeze-dried (CHRIST Alpha 1-4, B.Braun Biotech International) before further analysis of cellular composition. The supernatant was filtered through glass fibre filters (Whatman GF/C, 90 mm ⌀, nominal particle retention 1.2 μm) to remove possibly remaining cells, before transferring to 50 mL Falcon tubes. These were also stored at –20°C before determination of residual nutrient concentrations.

2.2 Characterization of yeast production wastewater

Analysis of micronutrients, heavy metals, total organic carbon (TOC) and dissolved organic carbon (DOC) in yeast production wastewater was performed by Almalab laboratory

(University of Helsinki, Lahti, Finland). For determining the concentrations of Na, K and Ca, the samples were diluted to 1 % with ultrapure water and treated with La-Ce reagent. They were then analysed using an atomic absorption spectroscopy unit (FAES Thermo M-Series). For determining the concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, P, Pb, Se, V and Zn, the samples were diluted to 1 % with ultrapure water and treated with nitric acid. They were then analysed using an inductively coupled plasma mass spectrometry unit (Elan 6000 ICP-MS). TOC and DOC concentrations were measured with a Teledyne-Tekmar Apollo 9000HS TOC analyser. The TOC measurement included particles below 0.8 mm in size.

Total suspended solids were measured by filtration onto pre-weighted glass fibre filters (Whatman GF/C, 47 mm ⌀, nominal particle retention 1.2 µm) that were then dried overnight at 105°C before weighing. Solids from filtered yeastwater were not measured as they had been filtered out. Measurements of COD, ammonium nitrogen (NH_4-N), total nitrogen (TN), phosphate phosphorus (PO_4-P) and total phosphorus (TP) were performed with Hach Lange kits as described in section 2.3.

BOD was determined from the yeastwater using an OxiTop Biological Oxygen Demand respirometer and an Oxitop control system (WTW, Weilheim, Germany) with an incubation period of seven days.

2.3 Analysis of COD and nutrients

Reduction in COD and nutrient (NH_4-N, TN, PO_4-P and TP) concentrations during the experiment were determined for all treatments. To accomplish this, COD and nutrient concentrations were first determined from the yeastwater and filtered yeastwater used to prepare the growth media and then from the supernatants obtained during biomass harvesting at the end of the experiment. For the experiments on synthetic media, starting nutrient concentrations were calculated based on the media recipes instead of determining

experimentally. Hach Lange kits (Hach Lange, Germany), a DR2800 spectrophotometer and a HT 200S heating block were used for the nutrient analyses following the manufacturer's instructions. COD and BOD are usually correlated in similar samples (Bourgeois *et al.* 2001), thus only COD was measured from the supernatants after biomass harvesting.

2.4 Biomass nitrogen analysis

To determine the biomass protein content, Kjeldahl nitrogen was determined from the freeze-dried biomass. Briefly, 0.05 g of biomass was weighed into 100 mL digestion tubes, two blank tubes were prepared for each set of 18 sample tubes. To each tube a tablet containing 3.5 g of K_2SO_4 and 0.5 g $CuSO_4$ was added before adding 20 mL of concentrated sulphuric acid. The tubes were then placed into an incubator set to 400°C for approximately two hours to complete the digestion of the biomass. The tubes were then allowed to cool to room temperature before adding 75 mL of ultrapure water while agitating gently. An automatic Kjeltec analyser (Tecator Kjeltec 2300) was used to perform the distillation and titration using a 35 % NaOH solution and a 1 % boric acid solution. The titration volume was recorded and used to calculate total Kjeldahl nitrogen.

The amount of nitrogen in the samples was calculated using the following formula:

$$m\ N(mg) = (V_{sample} - V_{blank}) \times c\ HCl \times M\ N$$

Where:

- m N is the mass of nitrogen in the sample (mg)
- V_{sample} is the volume of acid consumed in the titration for the sample (mL)
- V_{blank} is the volume of acid consumed in the titration for the blank samples (mL)
- c HCl is the concentration of the acid (mmol mL^{-1})
- M N is the molar mass of nitrogen (mg $mmoL^{-1}$)

The amount of protein was obtained by multiplying the amount of nitrogen with the conversion factor of 6.25 (Regulation (EU) No 1169/2011, annex I, at 10).

2.5 Cobalamin extraction and analysis

Extraction, purification and ultra-performance liquid chromatography (UPLC) analysis of cobalamin were performed with a method adapted from a paper by Chamlagain *et al.* (2015). The method is specific to the forms of the vitamin that are active in the human body.

Handling of samples and cobalamin extraction and purification were performed under yellow light or in vessels protected from direct light to avoid photodegradation of the vitamin. Freeze-dried biomass was used, 0.1 g was weighted into plastic extraction tubes and 10 mL of extraction buffer (8.3 mmol L^{-1} NaOH, 20.7 mmol L^{-1} acetic acid, pH 4.5) was added. To convert all cobalamin into the stable cyanocobalamin form, 100 µL of 1 % sodium cyanide solution was then added. The tubes were vortexed and then placed into a boiling water bath for 30 minutes. They were then cooled on ice and centrifuged for 10 minutes at 6900×*g* (Hermle Z 323, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was poured through a paper filter into 20 mL volumetric flasks and 5 mL of extraction buffer was added to the tubes to elute possibly remaining cyanocobalamin. The tubes were then vortexed and centrifuged again. The supernatant was added to the same volumetric flasks and the volume was adjusted to 20 mL using the extraction buffer solution. Finally, the sample solutions were transferred into plastic bottles and stored at –20°C.

Purification of cyanocobalamin was performed using immunoaffinity columns (3 ml, Easi-Extract® Vitamin B12, R-Biopharm, Rhone Ltd, UK) as described by Chamlagain *et al.* (2015). The frozen sample bottles were allowed to thaw in a 36°C water bath. The buffer was drained from the immunoaffinity columns and 10 mL of sample was slowly added. The sample bottle was washed with 9 mL of ultrapure water which was then added to the column. The remaining water was purged from the column by application of air. The sample was eluted into 3 mL of methanol followed by rinsing with another 0.5 mL of methanol and collected into clean tubes. The resulting eluate was evaporated under nitrogen flow in an incubator set to 50°C temperature. The sample residue was reconstituted into 300 µL of

ultrapure water and vortexed before filtering through 0.2 µm pore size syringe filters (13 mm, PTFE, VWR, USA) into UPLC vials.

The UPLC analysis method was adapted from the paper by Chamlagain *et al.* (2015) and performed with Waters Acquity UPLC equipment using a Waters ACQUITY UPLC HSS T3 1.8 µm column. Cyanocobalamin absorbance was detected at 361 nm wavelength with a photodiode array detector. Gradient elution was used with water and acetonitrile, both containing 0.025 % trifluoroacetic acid. The following mobile phase gradients (water:acetonitrile) were used for a 10 min run: 0–0.5 min (95:5), 0.5–5 min (60:40), 5–6 min (60:40), and 6–10 min a linear gradient from 60:40 to 95:5.

To determine presence of free cobalamin in the yeastwater, growth media and harvest supernatants, cobalamin was also measured from a small number of liquid samples. Cobalamin extraction and purification from liquid samples was performed as described above with the following modifications: Sample mass was 4 grams, 15 mL of extract was used for the immunoaffinity purification and the purified sample was reconstituted into 200 µL of water.

Waters Empower software was used for the collection and processing of chromatographic data.

2.6 Betaine extraction and analysis

For betaine extraction from the biomass, 0.05 g of freeze-dried biomass was weighted into plastic extraction tubes, 5 mL of methanol was added, and the tube was vortexed for 20 seconds. The tubes were placed in a platform shaker for 10 minutes and then sonicated (Branson 5510 ultrasonic cleaner, Emerson Co., USA) for 30 minutes. The tubes were then centrifuged for 10 minutes at 6900×*g* (Hermle Z 323, Hermle Labortechnik GmbH, Wehingen, Germany). One millilitre of supernatant was transferred into Eppendorf tubes and

evaporated under nitrogen flow. One millilitre of 1:10 water-acetonitrile solution was added to reconstitute the sample. Then the samples were filtered through 0.2 µm pore size syringe filters (13 mm, PTFE, VWR, USA) into analysis vials.

Yeastwater samples were diluted by transferring 500 µL of sample into a 5 mL volumetric flask and filling with acetonitrile. For liquid samples and diluted yeastwater samples, 200 µL of sample was added into an Eppendorf tube containing 1800 µL of acetonitrile. The tubes were vortexed briefly and 1 mL of sample was syringe filtered into analysis vials through 0.22 µm pore size syringe filters (13 mm, PTFE, VWR, USA).

Betaine content of samples was measured using high-performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD) using a method adapted from a paper by Zhao *et al.* (2013). Briefly, a Waters CORTECS HILIC 2.7 µm, 2.1×150 mm column was used. An isocratic elution was used with acetonitrile containing 30 mM ammonium acetate buffer (80:20, v:v) as the mobile phase. The flow rate was set to 0.4 mL min^{-1}. The column oven was set to 30°C, the drift tube temperature to 45°C and the gas pressure of the ELSD detector to 50 psi.

2.7 Statistical methods

Differences in nutrient and betaine reductions, biomass protein and cobalamin concentrations as well as DW and cell concentrations between treatments were tested with ANOVA using a post-hoc Tukey's test. Levene's test was used to test the heterogeneity of variances. Heterogenous variances were observed for data on NH_4-N reductions, cobalamin biomass concentrations and harvest day cell concentrations for *E. gracilis*. In these cases, differences were tested with Kruskal-Wallis test using a post-hoc Dunn’s test for pairwise differences.

3. Results

3.1 Characterization of yeast production wastewater

The concentrations of trace elements in the yeastwater are shown in Table 2. Out of the elements present in Hutner's medium only copper was below the detection limit. The high levels of iron and manganese originated from the groundwater well used by the yeast factory (personal communication, E. Varonen).

Table 2. Concentrations of selected elements in yeastwater prior to starting the growth experiment. Concentrations below the limit of quantification are indicated by '<LOQ' and the limit in question.

Element	μg L^{-1}
Na	2462
Mg	60 500
Al	<LOQ (900)
P	130 000
K	6934
Ca	1552 mg L^{-1}
V	54
Cr	59
Mn	3750
Fe	12 000
Co	58
Ni	895
Cu	<LOQ (230)
Zn	470
As	<LOQ (30)
Se	<LOQ (890)
Mo	56
Cd	<LOQ (4.3)
Pb	31

Nutrient contents of the growth media and the concentrations of solids, BOD_7, COD, DOC and TOC in yeastwater are presented in Table 3. Filtration of yeastwater lowered its phosphorus content, but the nitrogen content remained mostly the same. The ammonium concentration was higher after filtration. For Hutner's medium and betaine enriched Hutner's medium, the nutrient concentrations were not determined experimentally, but calculated based on the media recipes. The elevated total nitrogen concentration in betaine enriched Hutner's medium is due to the nitrogen present in the added betaine.

Table 3. Properties of the growth media prior to starting the growth experiment

	Yeastwater	Filtered yeastwater	Hutner's medium	Betaine enriched Hutner's medium
solids	11 g L^{-1}	not measured	not measured	not measured
DOC	19 g L^{-1} C	not measured	not measured	not measured
TOC	23 g L^{-1} C	not measured	not measured	not measured
COD	50 200 mg L^{-1}	44 400 mg L^{-1}	5560 mg L^{-1}	5040 mg L^{-1}
BOD_7	27 940 mg L^{-1}	24 840 mg L^{-1}	not measured	not measured
NH_4-N	13.1 mg L^{-1}	73.6 mg L^{-1}	466 mg L^{-1}	466 mg L^{-1}
TN	3620 mg L^{-1}	3708 mg L^{-1}	466 mg L^{-1}	1661 mg L^{-1}
PO_4-P	73.4 mg L^{-1}	53.6 mg L^{-1}	138 mg L^{-1}	138 mg L^{-1}
TP	165 mg L^{-1}	97.6 mg L^{-1}	138 mg L^{-1}	138 mg L^{-1}

3.2 Nutrient removal

Nutrient removal from the yeastwater was measured by comparing the measurements from the yeastwater and from the supernatants obtained during biomass harvesting. Results are shown percentage reduction compared to the starting concentrations (Fig. 1 a-e).

Reduction of COD did not differ within yeastwater ($P > 0.97$) or synthetic media ($P > 0.67$) treatments (Fig. 1-a).

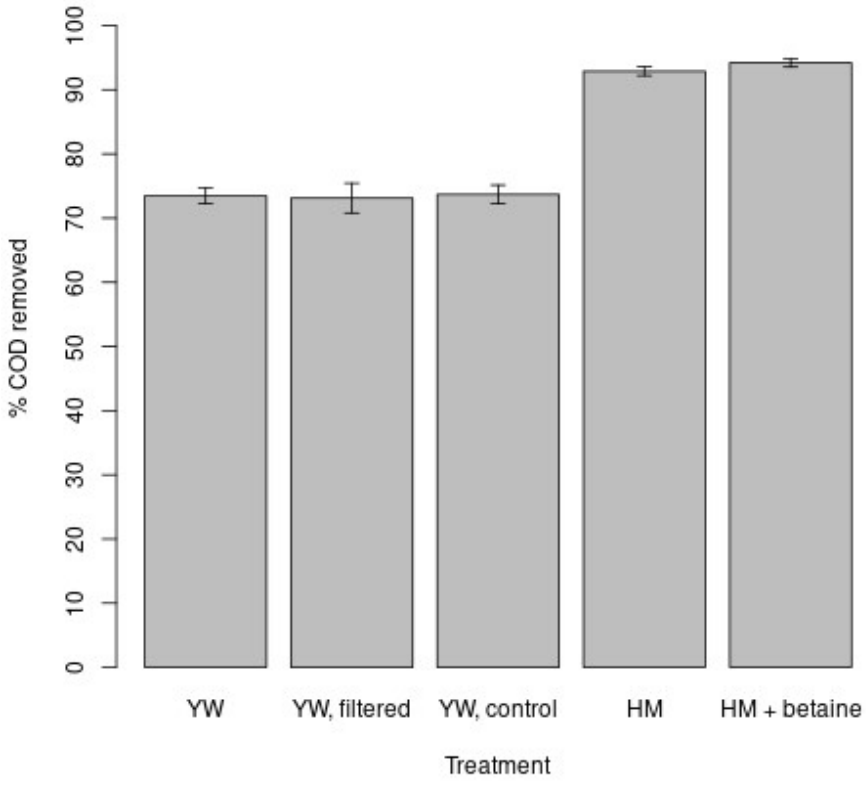

Figure 1-a. COD reduction as percentage of original; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

Concentrations of ammonium nitrogen were reduced more in filtered yeastwater compared to the control treatment ($P < 0.05$) (Fig. 1-b). In the Hutner's media experiments removal was close to 100 %.

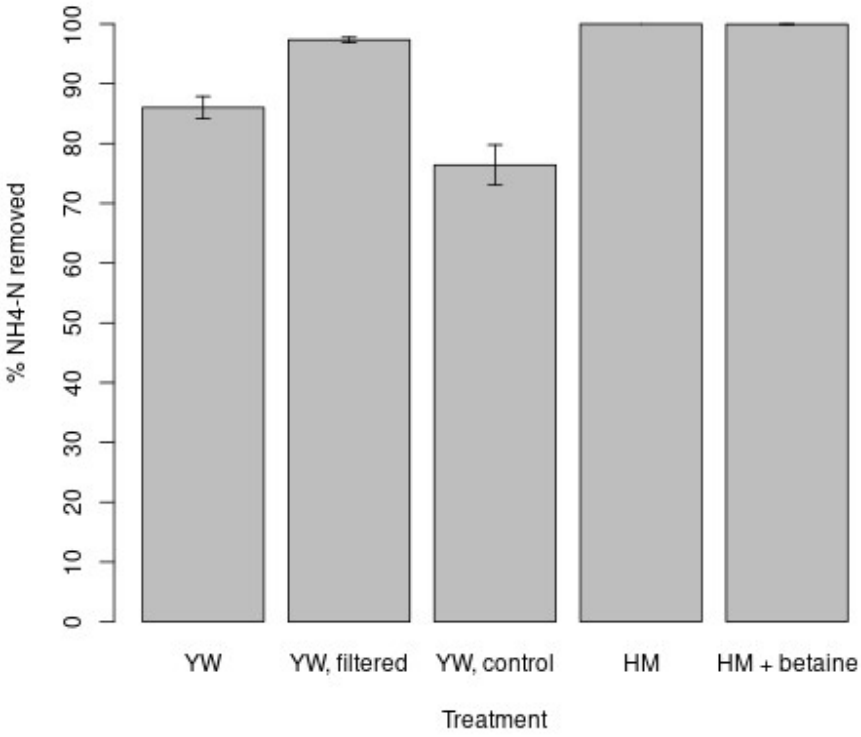

Figure 1-b. NH_4-N reduction as percentage of original; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

Concentrations of TN were reduced more in filtered yeastwater compared to unfiltered or control treatments, but the difference was not statistically significant (Fig. 1-c).

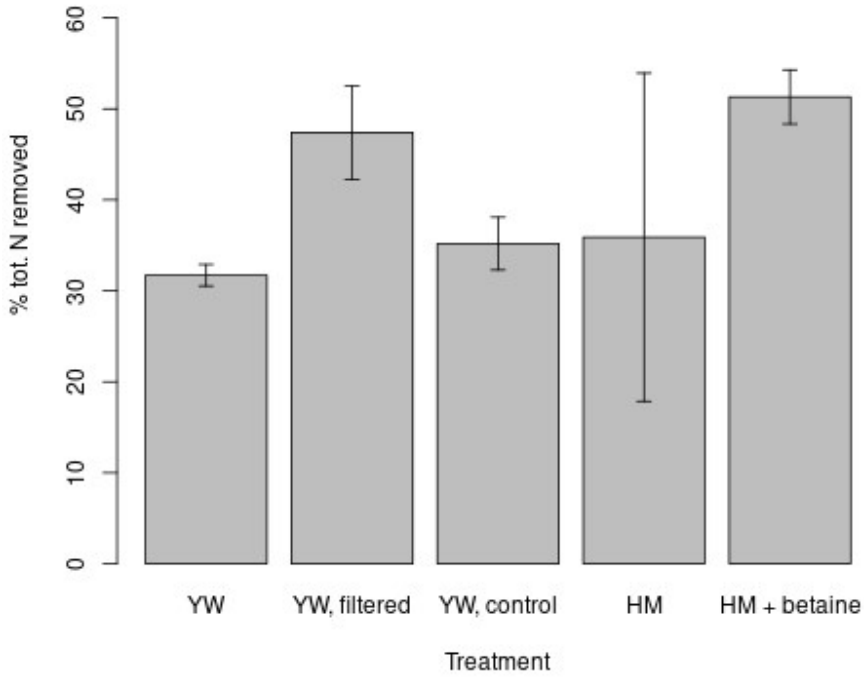

Figure 1-c. Total nitrogen reduction as percentage of original; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

PO_4-P concentrations were reduced more in betaine enriched Hutner's medium than in the standard Hutner's medium treatment ($P < 0.01$) (Fig. 1-d). No differences were observed between the yeastwater treatments as the removal efficiencies were close to 100 %.

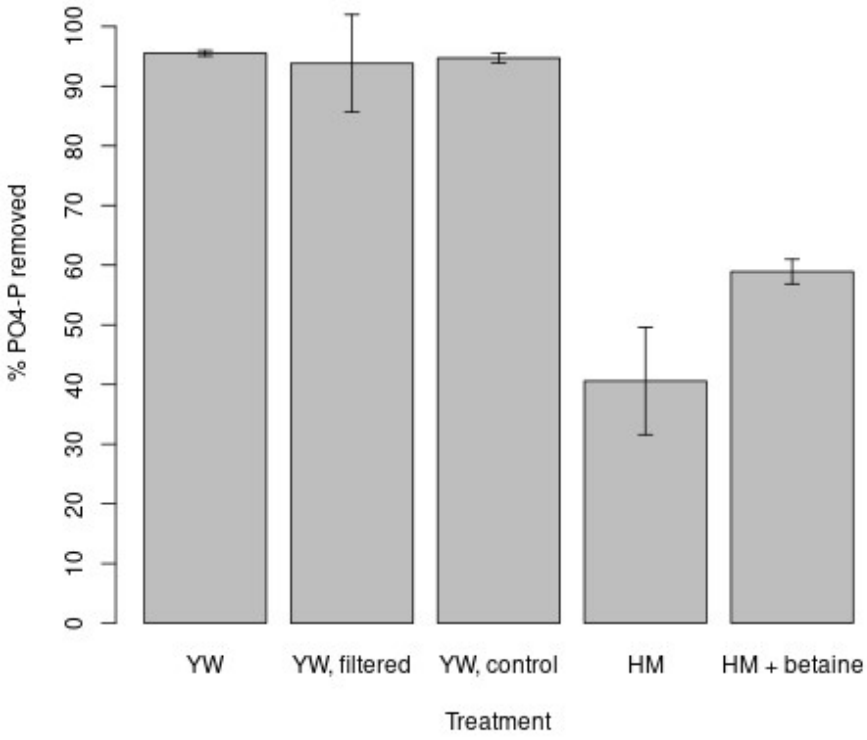

Figure 1-d. PO_4-P reduction as percentage of original; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

Similarly to PO_4-P concentrations, TP concentrations (Fig. 1-e) were reduced more in betaine enriched Hutner's medium than in the standard Hutner's medium treatment ($P < 0.001$). No clear difference was observed between the yeastwater treatments.

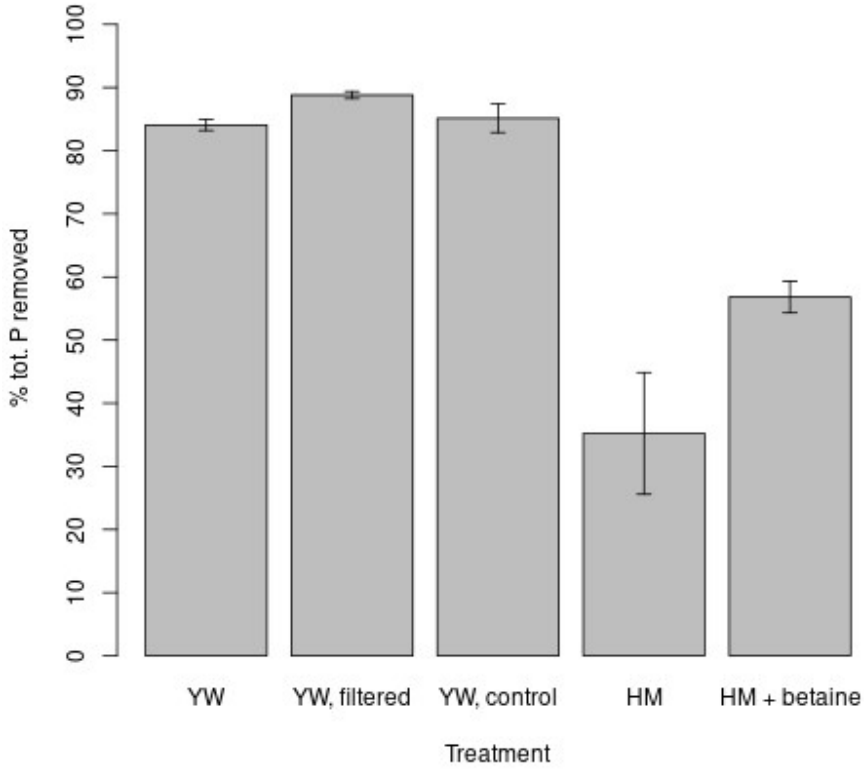

Figure 1-e. TP reduction as percentage of original; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

The culture media pH was monitored from the samples taken during the experiments (Fig. 2). All yeastwater experiments had a fairly high pH by the end of the experiments; 8.4 in yeastwater, 8.3 in filtered yeastwater and 8.6 in the control treatment. In both experiments carried out in Hutner's medium, conditions became very acidic with mean pH of the last day being 2.2 in Hutner's medium and 2.7 in betaine enriched Hutner's medium.

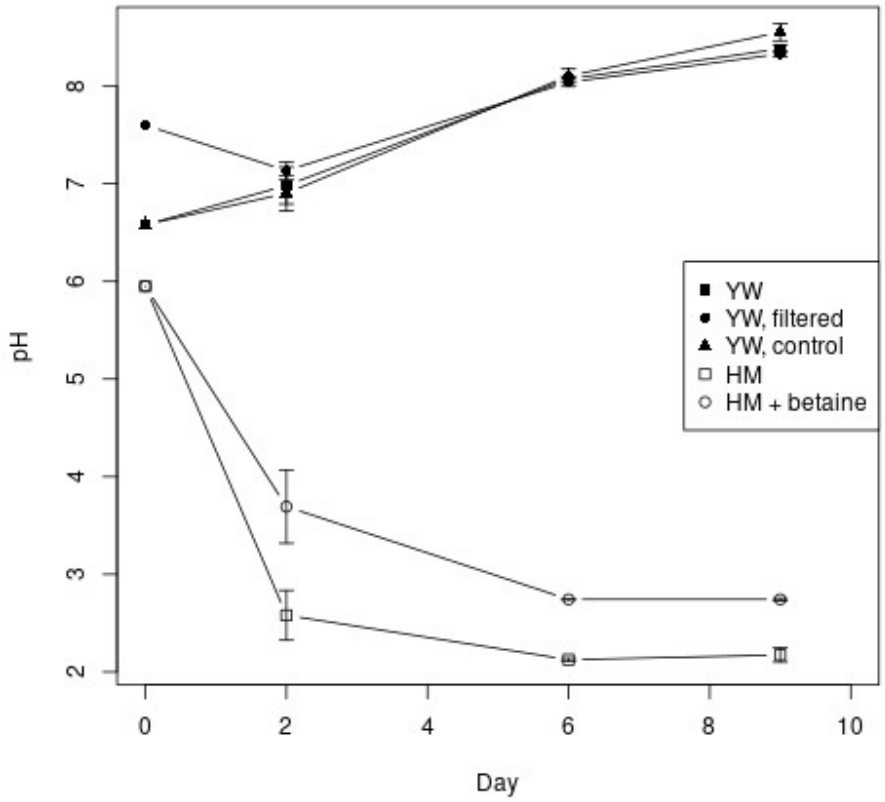

Figure 2. Development of pH in culture media during the experiments; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

3.3 Algal growth and biomass production

An average dry weight concentration of 2.2 g L^{-1} was reached in filtered yeastwater and in unfiltered yeastwater, while in the control experiment the mean concentration was 2.0 g L^{-1} (Fig. 3). However, the unfiltered yeastwater treatment the control experiment had a dry weight of 1.1 g L^{-1} at the start of the experiment due to the solids present in yeastwater. When the increase in DW during the experiment was calculated, the differences were more notable. An average dry weight increase of 1.9 g L^{-1} was found in filtered yeastwater. The DW

increase was lower (0.84 g L^{-1}) in yeastwater and significantly lower in the control experiment (0.86 g L^{-1}) ($P < 0.05$). A dry weight concentration of 3.1 g L^{-1} was reached in Hutner's media, while betaine enriched Hutner's media attained a significantly higher ($P < 0.001$) concentration of 7.1 g L^{-1}.

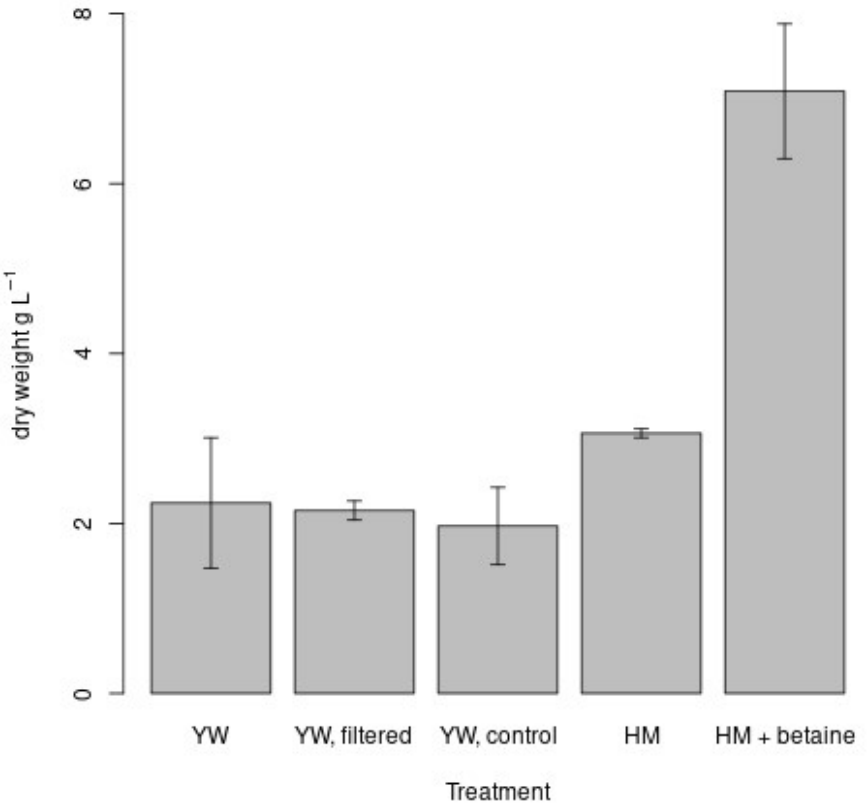

Figure 3. DW concentration on day of harvest; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates, error bars represent standard deviation.

Measurements of *E. gracilis* cell concentrations (Fig. 4) provided results similar those based on DW. While the starting concentration had been 300 cells μL^{-1}, at the end of the experiments the mean concentration was 340 cells μL^{-1} in yeastwater and 800 cells μL^{-1} in filtered yeastwater. No algal cells were detected in the control yeastwater treatment. In Hutner's medium the mean cell concentration was 1260 cells μL^{-1} and in betaine enriched

Hutner's medium 810 cells μL^{-1}. No statistically significant differences between the yeastwater and filtered yeastwater treatments or between the two Hutner's medium treatments could be established. This result is not in agreement with the DW measurements where betaine enriched Hutner's medium attained the highest growth.

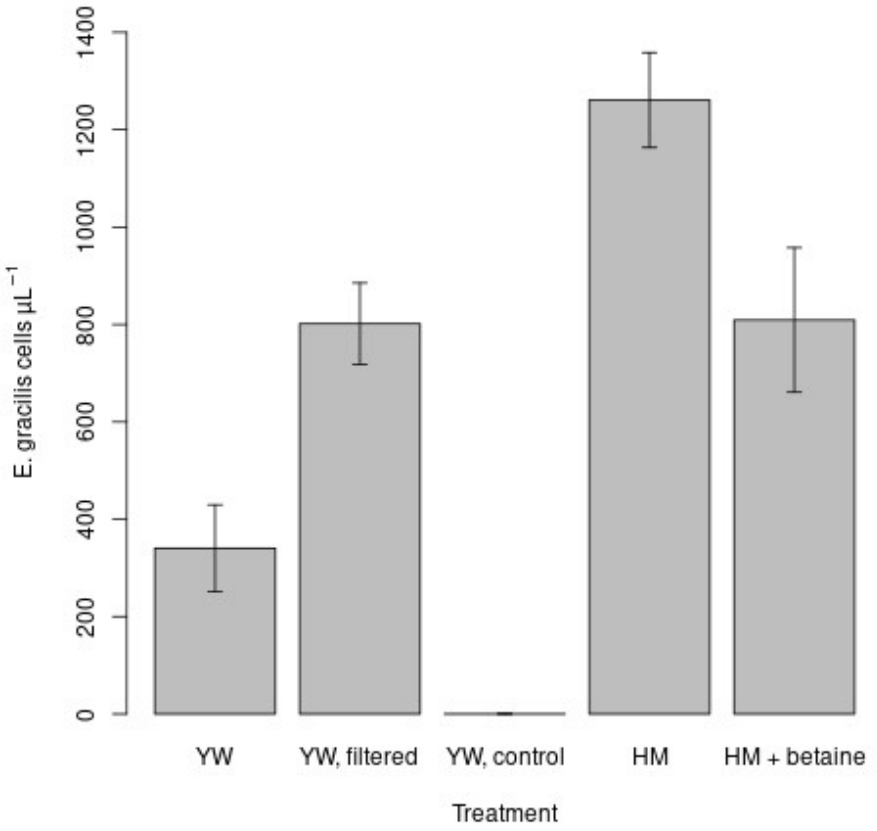

Figure 4. Cell concentrations of *E. gracilis* on day of harvest as measured by flow cytometry; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

Biomass concentrations in terms of DW peaked on day nine (Fig. 5) and cell concentrations on day six (Fig. 6). The higher biomass concentrations observed on day nine are likely an artefact resulting from the different sampling method used on that day. Instead of drawing samples with a syringe, the whole bottles were emptied.

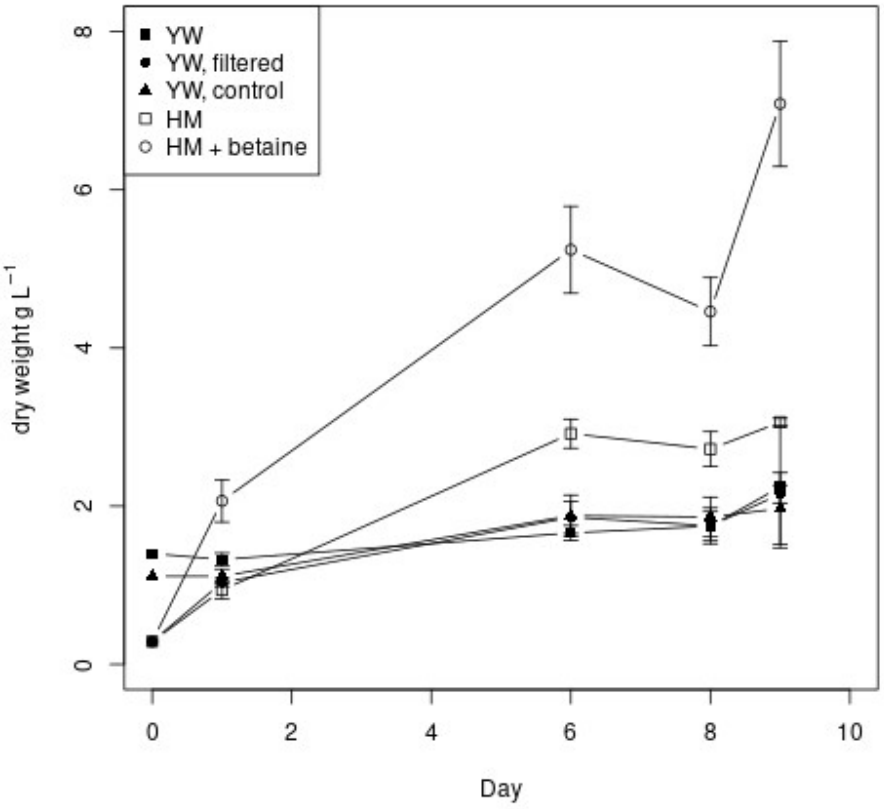

Figure 5. Dry weight over time; YW = yeastwater, HM = Hutner's medium; the symbols represent averages of the four replicates, error bars represent standard deviation.

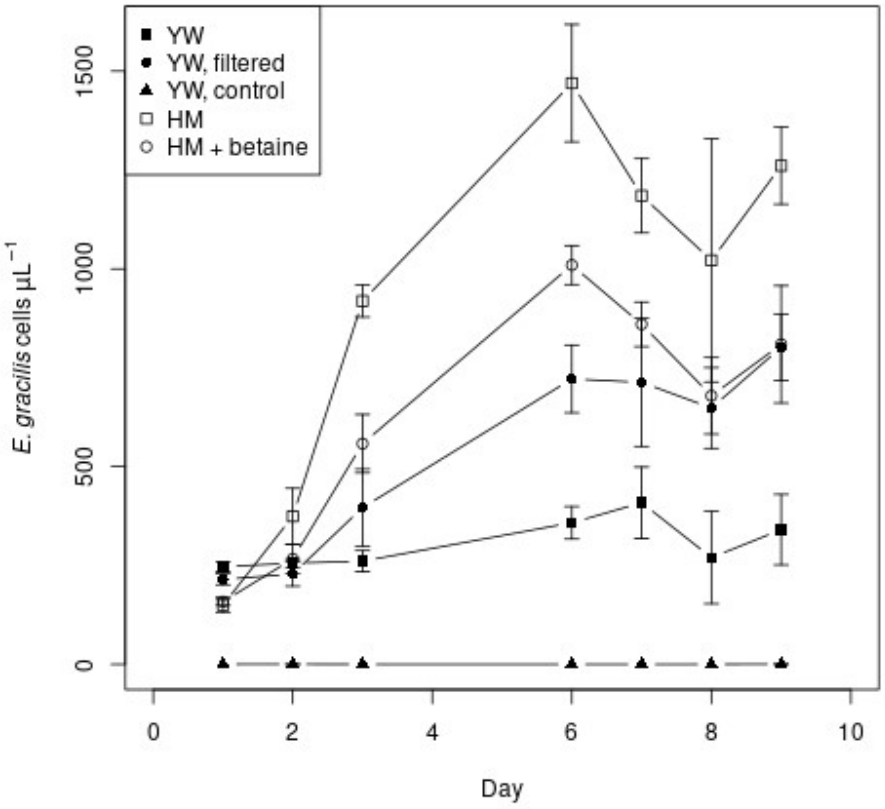

Figure 6. Cell concentrations of *E. gracilis* over time; YW = yeastwater, HM = Hutner's medium; the symbols represent averages of the four replicates, error bars represent standard deviation.

Cell concentrations of yeasts (Fig. 7) show, that in Hutner's medium and betaine enriched Hutner's medium experiments, the cultures were contaminated by yeasts.

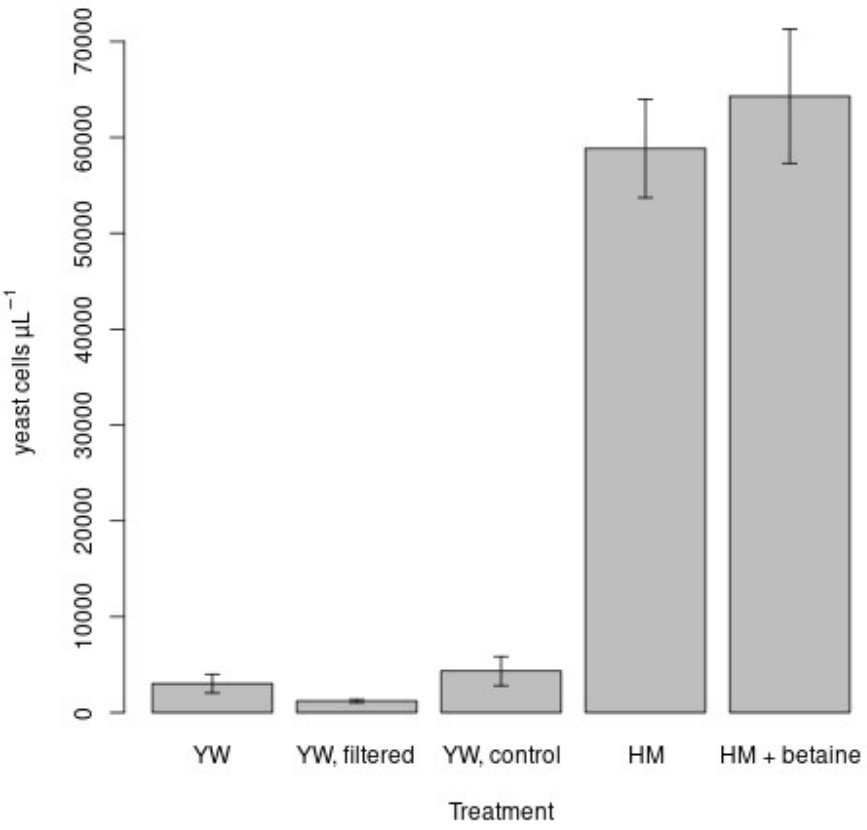

Figure 7. Cell concentrations of yeasts on day of harvest as measured by flow cytometry; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates; error bars represent standard deviation.

Yeast cell concentrations over time (Fig. 8) show a dramatic rise in Hutner's medium and betaine enriched Hutner's medium experiments. Data for Hutner's medium and betaine enriched Hutner's medium experiments on day six are missing.

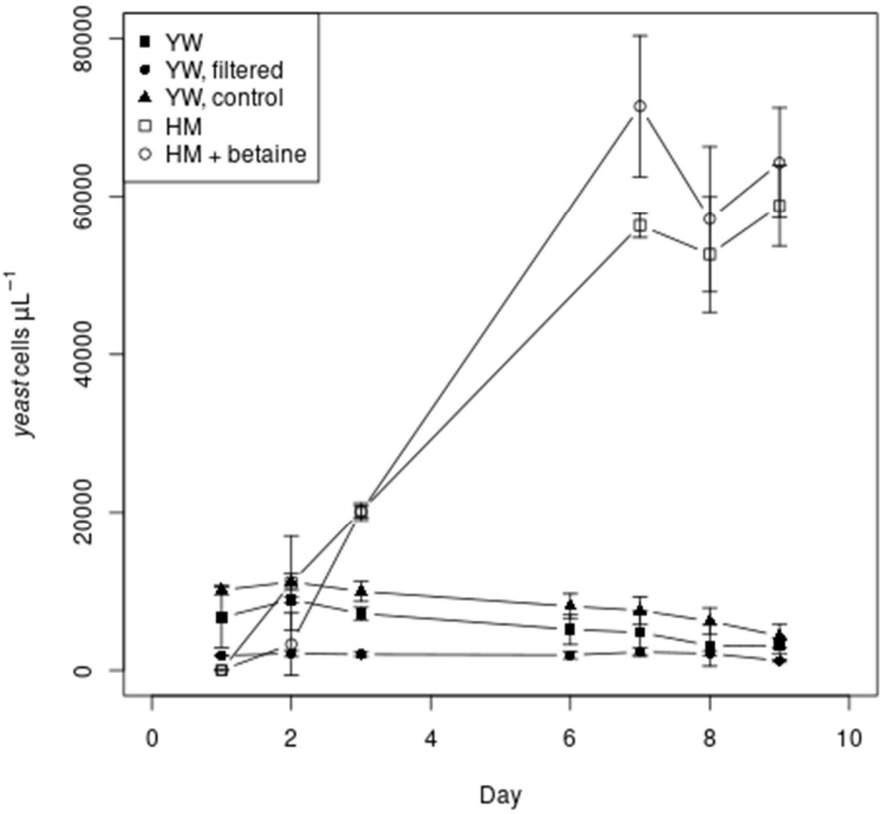

Figure 8. Cell concentrations of yeast-like particles over time; YW = yeastwater, HM = Hutner's medium; the symbols represent averages of the four replicates, error bars represent standard deviation.

3.3.1 Biomass nitrogen and protein content

The mean concentrations of Kjeldahl nitrogen were translated to protein concentrations with the conversion coefficient of 6.25 used for indicating protein content in foods (Regulation (EU) No 1169/2011, annex I, at 10) (Fig. 9). No significant differences in nitrogen content were found in the biomass from the different growth experiments.

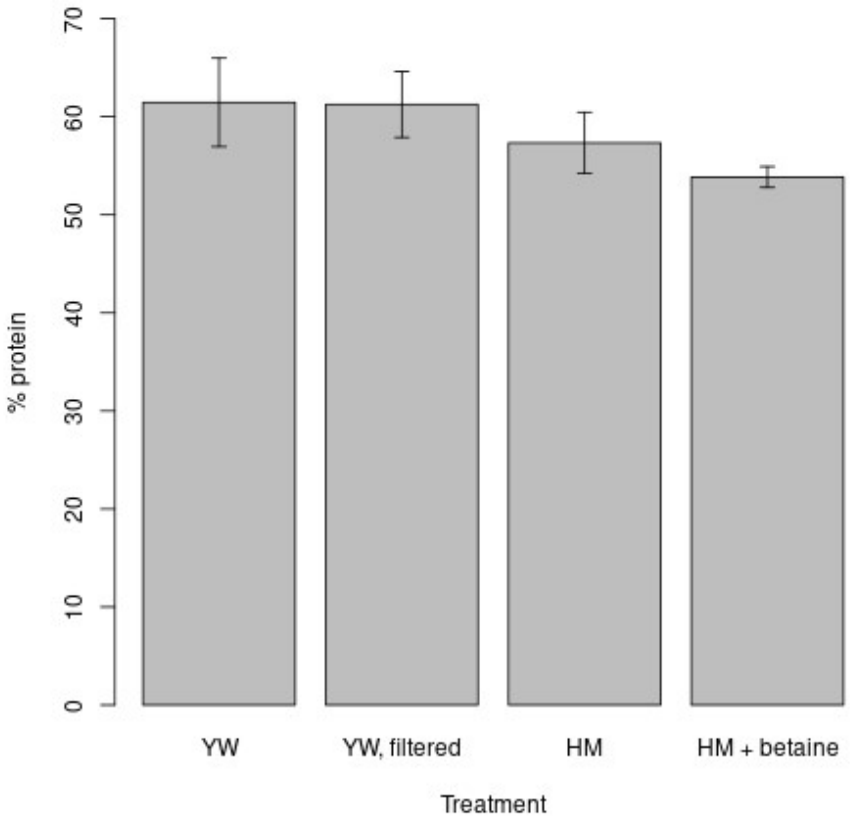

Figure 9. Total biomass protein as determined by Kjeldahl method; YW = yeastwater, HM = Hutner's medium; YW control treatment not analysed; bars represent means of four replicates, error bars represent standard deviation.

3.4 Betaine analysis

The betaine concentration of yeastwater was 6.6 g L^{-1} and that of filtered yeastwater 6.3 g L^{-1} which were used to calculate the concentrations in the final culture media. In betaine enriched Hutner's medium the measured mean betaine concentration was 6.4 g L^{-1} (Table 4).

Table 4. Concentrations of betaine in the culture media, harvest supernatants and harvested biomass. Data points with an asterisk represent means where betaine was not detected in all the replicates, leading to high uncertainty of the means

Treatment	Culture medium g L^{-1}	Harvest supernatant g L^{-1}	Biomass mg g^{-1}
Yeastwater	0.66	0.12*	0.87*
Filtered yeastwater	0.63	0.59	1.97*
Control yeastwater	0.66	0.52	4.87
Betaine enriched Hutner's medium	6.4	4.1	24.5
Inoculate	-	-	1.65*

Reduction of betaine concentrations in the growth media is shown as percentage reduction compared to starting concentrations (Fig. 10). In unfiltered yeastwater betaine concentrations were reduced by 82 %, significantly more than in filtered yeastwater (6.0 %) or in the control treatment (22 %) ($P < 0.01$).

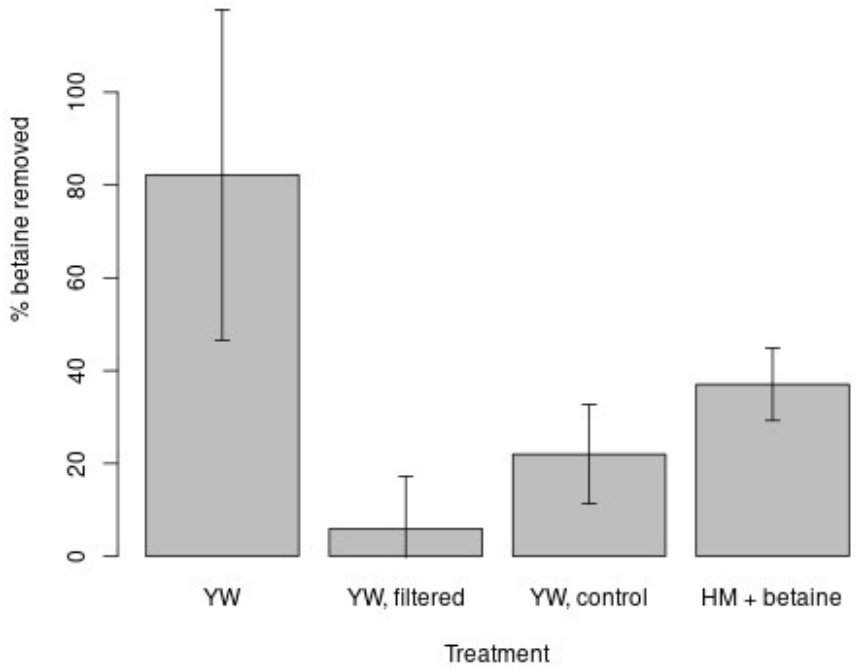

Figure 10. Betaine removal; YW = yeastwater, HM = Hutner's medium; HM treatment not analysed; bars represent means of four replicates, error bars represent standard deviation.

3.5 Cobalamin analysis

Cobalamin was detected in all biomass samples. Mean cobalamin concentrations of 20 µg g^{-1} in biomass from yeastwater and yeastwater control treatments were measured (Fig. 11). In biomass of filtered yeastwater treatments the mean concentration was significantly ($P < 0.05$) lower (4.5 µg g^{-1}). However, in all treatments excluding betaine enriched Hutner's medium, biomass cobalamin concentrations were higher than in inoculated biomass (3.3 µg g^{-1}). Concentrations in betaine enriched Hutner's medium remained significantly lower than in biomass from Hutner's medium experiment ($P < 0.05$) or inoculate biomass. Non-replicated measurements from yeastwater, filtered yeastwater and from the harvest supernatants of yeastwater and yeastwater control treatments showed concentrations below 0.005 µg g^{-1}.

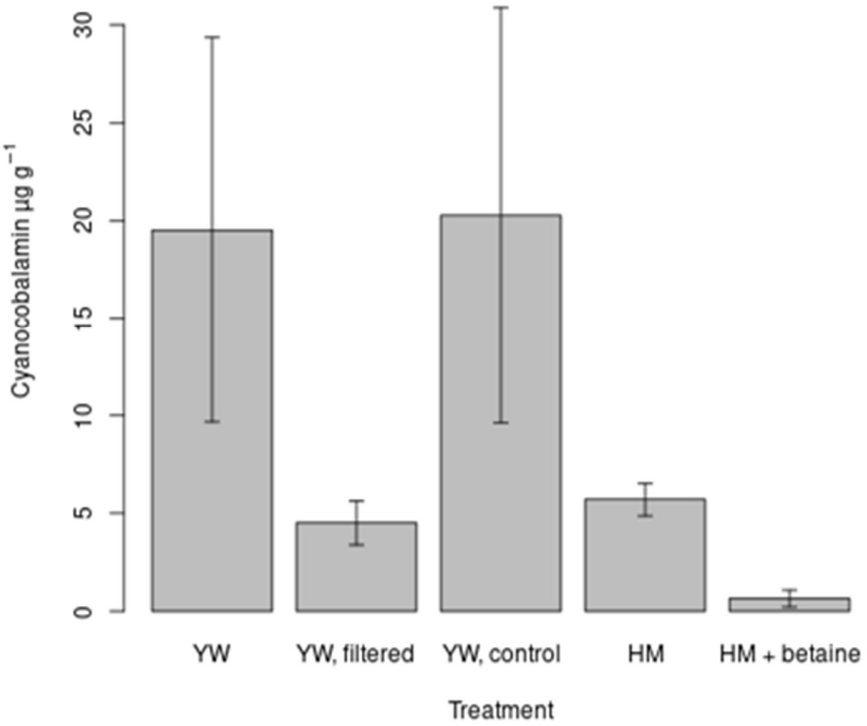

Figure 11. Cobalamin concentrations in biomass; YW = yeastwater, HM = Hutner's medium; bars represent means of four replicates, error bars represent standard deviation.

4. Discussion

4.1 Nutrient removal

Ammonium concentration was higher in filtered yeastwater compared to unfiltered yeastwater, which could be attributed to microbial activity during yeastwater handling and filtration. The removal of ammonium and total nitrogen was increased in filtered yeastwater, but not in yeastwater treatment compared to the control yeastwater treatment. Higher production of algal biomass in filtered yeastwater is the likely reason for this difference.

Any difference between yeastwater and yeastwater control treatments may be too small to be detected in this experimental setup, due to low biomass productivity in yeastwater. The number of yeast cells decreased in all of the yeastwater treatments during the experiment. However, it is possible that growth of bacterial biomass in the yeastwater control treatment was able to bind nutrients to the same degree as the alga in the yeastwater treatment.

Mahapatra *et al.* (2013) cultivated *E .gracilis* in domestic wastewater and found nutrient reductions of 98 % (NH_4-N), 93 % (TN), 85 % (PO_4-P) and 66 % (TP) in eight days. Tossavainen *et al.* (2018) cultivated *E. gracilis* in biowaste leachate and found nutrient reductions of over 70 % (COD), 97.5 % (NH4-4), over 90 % (TN), over 50 % (PO4-P) in ten days. Therefore, the phosphorus removal efficiency in our experiment was good but the removal of TN was quite low. Yeastwater has a N:P ratio of 22, which is high compared to common types of wastewater (Cai *et al.* 2013). Others have found that the optimal N:P ratio for maximizing biomass productivity of *Chlorella vulgaris* in wastewater was approximately 10 (Choi & Lee 2015). Phosphorus is therefore likely to be the limiting nutrient in yeastwater algal cultures. This is the most likely reason for the mediocre nitrogen removal efficiency and high phosphorus removal efficiency in the experiments.

Additionally, the nitrogen in yeastwater may be in recalcitrant forms, which is most likely, since part of the nitrogen in molasses is known to be bound in recalcitrant melanoidins and since easily used forms of nitrogen were likely used by the yeast culture. Sugar beet molasses is high in melanoidins, which are nitrogen containing, complex molecules (Coca *et al.* 2004) generally thought to be resistant to biological degradation. Cyanobacteria have successfully been used for melanoidin removal from wastewater (Kalavathi *et al.* 2001). Nonetheless, it is not surprising that in this experiment, nearly all ammonium was removed while removal of total nitrogen was much lower. Ammonium is the preferred form of nitrogen for microalgae (Maestrini *et al.* 1986) and it can also be removed by escaping into the air, especially at high pH (Nurdogan & Oswald 1995).

Regarding removal of COD, phosphate or total phosphorus, there was no observable difference between the different yeastwater treatments. For COD this is expected as it is a process mainly controlled by non-photosynthetic microbia and as air bubbling continuously supplied oxygen to the growth medium. Although this experiment cannot fully distinguish between algal nitrogen uptake and bacterial denitrification, the results suggest that *E. gracilis* is capable of taking up nitrogen from yeastwater and therefore reducing its concentrations.

4.2 Algal growth and biomass production

Peak cell concentrations occurred on the sixth day of cultivation with DW and cytometry measurements agreeing on this. A rise in concentrations is seen on day nine for all experiments, but this is likely due to the different sampling method used on that day. On harvest day, as the growth bottles were removed from the growth chamber it was possible to agitate them to a much greater degree than during the daily samplings. This led to more cells coming loose from the growth bottle walls and bottom.

Overall, the different yeastwater treatments reached similar biomass concentrations. However, the change in biomass concentration was higher in filtered yeastwater. Cell concentration measurements support this observation, indicating a significantly higher concentration in filtered yeastwater compared to the other treatments. The likely reason that DW alone was not a reliable estimate of productivity in this case, is the fact for not being filtered, the yeastwater growth medium contained a significant amount of solids already at the start of the experiment. Production of new algal biomass and disintegration of the yeastwater solids likely occurred in parallel, confounding the DW measurements. Furthermore, DW increased during the experiment also in the yeastwater control treatment, suggesting that new microbial biomass was produced. In our case, however, the cytometry measurements support the observation that growth of *E. gracilis* in yeastwater was less than half of that in filtered yeastwater.

Visual observations indicated higher turbidity of the yeastwater medium, which may have contributed to lower algal productivity due to lower light penetration. Higher wastewater turbidity correlates negatively to algal growth rates (Wang *et al.* 2010). Candido and Lombardi (2017) also noted the beneficial effect of filtering when using ethanol production vinasse for cultivation of *Chlorella vulgaris*. In addition to turbidity, other possible explanations are differences in the microbial communities and the presence of additional growth inhibiting organic compounds in the yeastwater growth medium.

The cytometry measurements of yeast cell concentrations reveal that both experiments carried out in Hutner's medium were contaminated by excessive growth of yeasts. This makes the DW measurements unreliable for quantifying productivity of *E. gracilis* in the Hutner's medium experiments. Cytometry measurements of *E. gracilis* cells remain useful. Furthermore, yeast cell concentrations differed only to a minor degree between Hutner's medium and betaine enriched Hutner's medium experiment, while the difference in DW was very large. Therefore, the assumption that a higher *E. gracilis* biomass was truly achieved in betaine enriched Hutner's medium is not totally unwarranted. However, the very high DW concentration of 7.1 g L^{-1} in betaine enriched Hutner's medium treatment should be considered an artefact caused by the yeast contamination.

DW and cytometry measurements provided conflicting data for Hutner's medium and betaine enriched Hutner's medium experiments. Cell concentrations in Hutner's medium were much higher than in betaine enriched Hutner's medium, DW was lower. The only ways that betaine enriched Hutner's medium differed from Hutner's medium were the lack of cobalamin and the presence of betaine. *E. gracilis* can utilize glycine as a source of nitrogen (Richter *et al.* 2015). In yeastwater media this is unlikely to matter as nitrogen was plentiful, but in the betaine enriched Hutner's medium treatment, glycine may have been released from betaine and this might have contributed to the increased growth of the alga compared to growth in Hutner's medium. The greater removal of phosphorus in betaine enriched Hutner's medium also points towards this explanation.

The difference in DW could also be due to larger mass of individual cells in betaine enriched Hutner's medium or the yeast contamination contributing to the DW. However, yeast cell concentrations were similar in the two treatments. Cobalamin deficiency has been shown to lead to higher cell volumes in *E. gracilis* (Shehata & Kempner 1978), which is caused by blocked DNA replication and cell division (Lefort-Tran *et al.* 1987). The effects of salt stress caused by the added 0.18 M Cl- from betaine-HCl and Na+ used to adjust pH could have had a similar effect because salt stress can increase cell size in *E. gracilis* (González-Moreno *et al.* 2006).

It is known that in the right conditions, *E. gracilis* can accumulate nutrients faster than suggested by the cell division rate, storing them intracellularly and using them later (McCalla 1963, Crimp *et al.* 2017). Betaine enhances cellular metabolism by acting as a methyl group donor or osmoprotectant and therefore allows enhanced storage of nutrients intracellularly, which could have contributed to the increased cell mass. However, the measurements of biomass nitrogen concentrations do not support this hypothesis since no differences between protein concentrations in the different treatments were found. In addition to nutrients, *E. gracilis* can also accumulate storage compounds such as paramylon (Suzuki & Suzuki 2013), lipids (Coleman *et al.* 1988) and wax-esters (Inui *et al.* 1982).

At the yeast factory, thiamine and biotin are *E.* gracilis is auxotrophic (i.e. requires it for its growth) in terms of thiamine. Thiamine was added to both Hutner's media treatments, but it was also likely present in yeastwater since it is added to the culture medium at the yeast factory to promote growth of the yeast (personal communication E. Varonen). The concentrations of thiamine were not measured in this study, but it is unlikely that yeastwater would have been lacking in thiamine.

4.3 Protein production

Biomass protein concentrations did not differ significantly between the experiments. This is surprising since the total nitrogen content of betaine enriched Hutner's medium was higher than that of Hutner's medium. Possibly the presence of betaine allowed the cells in betaine enriched Hutner's medium to accumulate more storage compounds low in nitrogen such as lipids or starches, therefore lowering the proportion of protein.

The reported protein content was calculated from total nitrogen content using the conversion factor of 6.25 typically used for foods. It might not give an accurate estimate of protein content as different conversion factors have been proposed for microalgae (González López *et al.* 2010). The correct conversion factor depends on the concentrations of non-protein-nitrogen, such as nucleic acids, chlorophylls and inorganic nitrogen (Lourenço *et al.* 2004) in the biomass. The proportion of non-protein nitrogen varies heavily based on algal species and growth phase among other things (Lourenço *et al.* 2004). Therefore, accurate estimation of protein content of the produced biomass would require more advanced analyses than those performed in this study, such as the Lowry protein assay (Lowry *et al.* 1951) or determinations of nucleic acid concentrations.

4.4 Cobalamin and betaine

Concerning cobalamin production, lower concentrations in filtered yeastwater suggest that filtration of the yeastwater removed some component that promotes cobalamin synthesis. Whether this was an organic compound promoting microbial cobalamin synthesis, or a microbial population is not known. However, as cobalamin is only produced by a few strains of microbia, it is possible that they were filtered out of the growth medium even though the filters used were coarse enough to allow bacterial cells through. Production of cobalamin did not differ significantly between yeastwater and the yeastwater control treatments. This suggests that, if algal symbiosis has an effect on bacterial cobalamin production, the effect is

likely to be very small. It has been shown that under photoautotrophic conditions, the bacterial symbiont is dependent on organic carbon produced by the microalga (Kazamia *et al.* 2015). In yeastwater, however, organic carbon was plentiful and the effect of microalgae on bacterial cobalamin synthesis would have been difficult to demonstrate.

In the experiments performed in Hutner's medium betaine did not promote the bacterial production of cobalamin but seemed to have the opposite effect. The cobalamin concentrations were lower in biomass produced in betaine enriched Hutner's medium whether expressed as proportion of the biomass or of the culture liquid volume.

Interestingly, betaine was consumed to a high degree only in the yeastwater treatment, while in filtered yeastwater and yeastwater control treatments only a minor reduction was measured. This means that neither the presence of algae nor the presence of other micro-organisms can alone explain betaine consumption. It could be hypothesised that a symbiotic relationship between the alga and other micro-organisms is involved in the use of betaine or that the alga being more stressed in yeastwater compared to filtered yeastwater caused the difference. The connection of betaine consumption and cobalamin production does not look likely based on these results, but neither can bacterial-algal interaction be ruled out. Betaine did not accumulate in the algal biomass to a significant degree. This is evidenced by the fact that in all treatments, betaine concentrations in the solution remained nearly two orders of magnitude higher compared to those in biomass.

4.5 Final words

The final biomass concentration of 1.9 g L^{-1} in filtered yeastwater shows promise for the use of this nutrient feedstock in algal culture and warrants further study especially if scalable ways to reduce its turbidity are found. A possible use of yeastwater is the fortification of nutrient feedstocks that are poor in nitrogen but high in phosphorus, combining the strengths

of different raw materials. Further research is needed to determine whether betaine enhances the accumulation of storage compounds or intracellularly stored nutrients in microalgae.

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7. Appendices

Appendix A: Media composition in Hutner's medium and betaine enriched Hutner's medium experiments

Chemical	**Hutner's medium (g L^{-1})**	**Betaine enriched Hutner's medium (g L^{-1})**
Glucose	5.0	5.0
$(NH_4)_2SO_4$	2.0	2.0
KH_2PO_4	0.4	0.4
$(NH_4)_2HPO_4$	0.2	0.2
$MgSO_4 \cdot 7H_2O$	0.5	0.5
$CaCl_2$	0.2	0.2
H_3BO_3	0.0144	0.0144
Thiamine	0.0025	0.0025
Betaine-HCl	-	12.4
	mg L^{-1}	mg L^{-1}
$ZnSO_4 \cdot 7H2O$	44	44
$MnSO_4 \cdot H2O$	11.6	11.6
$NaMoO_4 \cdot 2H_2O$	3.0	3.0
$CuSO_4 \cdot 5H_2O$	3.2	3.2
$CoCl_2 \cdot 6H_2O$	2.8	2.8
$(NH_4)_2SO_4Fe(SO_4)_2 \cdot 6H_2O$	11.4	11.4
EDTA	10	10
	µg L^{-1}	µg L^{-1}
Cyanocobalamin	20	-